REMARKS/ARGUMENTS

Claims 1-9, 13-32, directed to the following species: 1) BCG and interferon-γ, or LPS, TNF-α as maturing agent, and 2) CD86 or CD80 co-stimulatory molecule have been examined in the instant application.

Applicant again notes that all of the species should be considered together in the present application and respectfully request the Examiner further reconsider the need for restriction. In the present response all remarks are directed to the currently elected species although certain claims have not been amended to cancel the non-elected subject matter. Should the generic claims be found allowable, Applicant respectfully requests rejoinder of a reasonable number of non-elected species as set forth in M.P.E.P. § 821.04.

The prior rejections under 35 U.S.C. § 102 and § 103 have been withdrawn in view of amendments made to the claims in an earlier response. The following new rejections have been made.

Claims 1 and 5 have been amended to point out the claimed method with greater particularity. The amendment adds no new matter and does not limit the scope of the claim in any way.

The Examiner is respectfully requested to reconsider the pending claims in view of above amendments and the remarks below.

Rejections Under 35 USC § 102:

Claims 1 through 3, and 5 and 13 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Triozzi *et al.*. *Cancer* 89:2646-2654, 2000, and as evidenced by Labeur *et al.*. *J. Immunol.* 162:168-175, 1999. In particular, the Examiner has alleged that Triozzi *et al.* teach intatumoral injection of dendritic cells (DCs) derived *in vitro* in patients with metastatic melanoma or breast cancer. Triozzi *et al.* is believed by the Examiner to teach that DCs

obtained from isolated, autologous peripheral blood by treatment with GM-CSF and IL-4 and that the generated DCs express the co-stimulatory molecules CD80 and CD86, and low numbers of CD83. The Examiner further alleges that Triozzi *et al.* teach that tumor regression and tumor infiltration lymphocytes are induced in the treated melanoma and breast cancer patients. In addition, the Examiner has alleged that the DCs taught by Triozzi *et al.* are partially matured *in vitro* as evidenced by Labeur *et al.* wherein Labeur *et al.* teach that supplementing GM-CSF with IL-4 significantly enhanced DCs differentiation leading to an intermediate degree of maturation. The Examiner further alleges that Labeur *et al.* teach that DCs matured with GM-CSF and IL-4 have the ability to pick up antigen *in vitro* and process that antigen *in vivo* citing to Figure 3 on page 172 and Figure 4 on page 173. It is the opinion of the Examiner that DCs that are capable of picking up and processing of antigen are partially mature DCs as compared with those DCs that are terminally matured that lose the ability to pick up and process antigen.

The Examiner alleges that although Triozzi *et al.* does not explicitly teach that the generated DCs are partially mature, the claimed DCs appear to be the same as the prior art DCs. Therefore, as the method of the prior art comprises the same method steps as claimed in the instant invention using the same composition, the claimed method is anticipated because the method will inherently lead to the claimed effects.

Applicant strongly disagrees with the allegations and assertions of the Examiner, but to further expedite prosecution claim 1 has been amended to recite "[a] method for producing an anti-tumor immune response comprising administration to an individual with a cancerous tumor a cell population comprising partially mature dendritic cells that have been induced to mature *in vitro*, wherein the partially matured dendritic cells take up and process antigen *in vivo* and are enabled to induce an anti-tumor immune response subsequent to administration to the individual". Such amendment is believed to point out with greater particularity the method comprises administering an individual partially mature dendritic cells that have been induced to mature *in vitro* and that contact with antigen occurs *in vivo* subsequent to administration.

Further, Triozzi *et al.* do not use partially mature dendritic cells in the methods disclosed, but instead use immature dendritic cells differentiated from monocytes by culture in GM-CSF and IL-4. This method of generating immature dendritic cells from monocytes is well known to the skilled artisan. For example, Sallusto and Lanzavecchia, *J. Exp. Med.* 179:1109-1118, 1994, clearly demonstrated that monocytes when cultured in the presence of GM-CSF and IL-4 differentiate into immature dendritic cells. A copy of the reference is attached for the convenience of the Examiner.

Labeur *et al.* cited by the Examiner does not conflict with the teaching of Sallusto and Lanzavecchia. In particular, Labeur *et al.* induce the differentiation of dendritic cells from murine bone marrow dendritic cell precursors. It is well known in the art that the cytokines necessary to induce the differentiation of human immature dendritic cells and/or human mature dendritic cells from human monocytic dendritic cell precursors are different from those necessary to induce differentiation of murine immature dendritic cells and/or murine mature dendritic cells from murine bone marrow dendritic cell precursors. Labeur *et al.* demonstrate that bone marrow dendritic cell precursors from mice are induced to differentiate into immature dendritic cells by culture in GM-CSF alone. Culture of the bone marrow dendritic cell precursors in GM-CSF and IL-4 induced the cells to differentiate and mature as measured by cell surface phenotype and the substantial reduction in phagocytosis and endocytosis. The phenotype of dendritic cells produced by Labeur *et al.* by culture in GM-CSF and IL-4 differ only in the stimulus of mixed lymphocyte reactions and efficiency of in vitro peptide presentation when compared with bone marrow dendritic cell precursors cultured in the presence of GM-CSF and IL-4 with TNF-α, LPS or CD40L. Labeur *et al.* specifically state that IL-4 "is a potent enhancer of mouse DC maturation". See page 173, right column, last paragraph, lines 6-7. As such, Labeur *et al.* does nothing to support the characterization by the Examiner of the dendritic cells used by Triozzi *et al.* being partially mature dendritic cells.

In addition, the Examiner has noted that "fully mature DCs exposed to GM-CSF plus IL-4 and CD40L, taught by Labeur et al(Labeur et al, abstract), that have the ability to pick up and process antigen, are interpreted as partially matured DCs. Applicant has reviewed the

abstract of Labeur *et al.* and only find the following statements regarding intermediate dendritic cell maturation: "Whereas cells cultured in GM-CSF alone were functionally immature, cells incubated in CD40L or LPS were mature BmDC, as evident by morphology, capacity to internalize Ag, migration into regional lymph nodes, IL-12 secretion, and alloantigen or peptide Ag presentation in vitro. The remaining cultures exhibited intermediate dendritic cell maturation." See Labeur *et al.* abstract, lines 6-9. Applicant strongly disagrees that this statement supports the Examiner's conclusion that the DCs produced by Labeur *et al.* have the ability to pick up and process antigen and therefore should be interpreted to be partially mature DCs. In fact, Labeur *et al.* teach that bone marrow DCs induced by culture in GM-CSF and IL-4 do not retain the ability to take up and process antigen. Table II, at page 171, clearly shows that BmDCs cultured in GM-CSF and IL-4 have a substantially reduced ability to uptake antigen and have essentially the same capacity for phagocytosis as those BmDCs cultured in the presence of GM-CSF and IL-4 plus LPS or CD40L. At page 171, right had paragraph beginning at line 1 of the text, the authors explain the data as follows: "Incubation of cells with IL-4 resulted in a marked down regulation of FITC-*E. coli* uptake. Further addition of Flt3L, TNF-α, CD40L, or LPS did not have additional effects on phagocytosis". As such, even if the culture of monocytic dendritic cell precursor cells in GM-CSF and IL-4 could be directly compared with the culture of murine bone marrow dendritic cell precursor cells in GM-CSF and IL-4, the teachings of Labeur *et al.* are not supportive of the conclusion of the Examiner that the dendritic cells of Labeur *et al.* or Torizzi *et al.* are partially mature dendritic cells as defined and used in the present application and claims.

Applicant believes that the claims 1-3, 5 and 13 are not anticipated by Torizzi *et al.* in view of Labeur *et al.* As such, Applicant respectfully requests the Examiner to reconsider and withdraw the rejection of claims 1-3, 5 and 13 as being anticipated by Torizzi *et a.* in view of Labeur *et al.*

Rejections Under 35 USC § 103:

Claims 2 and 4 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Triozzi *et al.* (above) in view of Labeur *et al., J. Immunol.* 162:168-175, 1999, *supra*, in view of Murphy *et al.* (US 5,788,963). The Examiner alleges that Triozzi *et al.* and Labeur *et al.* do not teach that: 1) DCs are obtained from skin, spleen, bone marrow, thymus, lymph nodes, umbilical cord blood, and 2) DCs are obtained from a healthy individual HLA-matched to the individual to be treated. Further, the Examiner has alleged that Murphy *et al.* teach isolation of DCs fro prostate cancer therapy, where DCs are obtained from any tissue where they reside, including from the skin, the spleen, bone marrow, lymph nodes, and thymus as well as the circulatory system including blood, lymph and umbilical cord blood. The Examiner further, characterizes Marphy *et al.* as teaching that DCs can be obtained from a healthy individual HLA matched to the patient, because patients previously treated with radiation or chemotherapy often are not able to provide sufficient or efficient DCs. In addition, the Examiner notes that Murphy *et al.* teach that $CD8^+$ T cells, after interaction with antigen presenting cells, which express MHC class I or class II molecules associate with the antigen, are sensitized and capable of killing any cells that express the specific antigen associated with matching MHC class I molecules.

As such, the Examiner has alleged that it would have been *prima facie* obvious for one of ordinary skill in the art at the time the invention was made to obtain DCs taught by Triozzi *et al.* and Land Labeur *et al.* from skin, spleen, bone marrow, thymus, lymph nodes, umbilical cord blood, as taught by Murphy *et al.* to increase the number of available sources for making DCs. Further, the Examiner has alleged that it would have been obvious to replace the DCs obtained from the individual to be treated, taught by Triozzi *et al.* and Labeur *et al.* with DCs isolated from a healthy HLA matched individual as taught by Murphy *et al.* to increase the number of available DCs.

Applicant strongly disagrees with the allegations and assertions of the Examiner. As above, Triozzi *et al.* do not teach the administration of partially mature DCs, but instead teach the administration of immature DCs differentiated from monocytic dendritic cell precursors by

the standard method of culturing the monocytic dendritic cell precursors in the presence of GM-CSF and IL-4. Labeur *et al.* do not add anything to the disclose of Triozzi *et al.* that discloses or suggests either the administration of partially mature dendritic cells to a patient or the use of partially mature DCs isolated or differentiated from precursor cells isolated from any source. As above, Triozzi *et al.* teach that murine bone marrow dendritic cell precursors cultured in the presence of GM-CSF and IL-4 differentiate into a mature dendritic cell that have a significantly reduced ability to up take and process soluble antigen. The dendritic cells produced by the method of Labeur *et al.* do differ in their ability to induce an anti-tumor response when compared with other bone marrow dendritic cell precursor maturation agents, such as LPS or CD40L, but not in the ability to up take and process antigen.

The addition of Murphy *et al.* adds nothing to provide the missing elements from Triozzi *et al.* and/or Labeur *et al.* when consider either alone or in any combination. Therefore, as the Examiner has failed to establish a *prima facie* case for obviousness Applicant respectfully requests that the Examiner reconsider and withdraw the rejection of claims 2 and 4 as being unpatentable over Triozzi *et al.* in view of Labeur *et al.*, and further in view of Murphy *et al.*

Claims 6 through 9 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Triozzi *et al.* (above) in view of Labeur *et al. J. Immunol.* 162:168-175, 1999 (*supra*) and further in view of US 20050059151 (Bosch *et al.*), and Chakraborty *et al., Clin. Immunol.* 94:88-98, 2000). The teachings of Triozzi *et al.* and Labeur *et al.* as alleged by the Examiner are set forth above. In addition, the Examiner asserts that Triozzi *et al.* and Labeur *et al.* do not teach the use of BCG and interferon γ for maturing dendritic cells. Further, the Examiner admits that Triozzi *et al.* and Labeur *et al.* do not teach 1) BCG comprising whole BC, cell wall constituents of BCG, BCG-derived lipoarabidomannans, or BCG component, 2) the BCG is heat inactivated BCG or formalin-treated BCG, and 3) the effective amount of BCG and the effective amount of interferon γ.

The Examiner has combined the teachings of Bosch *et al.* which are alleged to be the maturing of DCs such as those previously exposed to GM-CSF plus IL-4, with interferon γ

and BCG to promote DC production of IL-12 and the reduction of the production of IL-10 thereby priming the mature dendritic cells for a Th-1 response with the teachings of Triozzi *et al.* and Labeur *et al.* Bosch is also alleged by the Examiner to teach 1) the effective amounts of BCG, 2) the effective amounts of interferon γ, 3) use of whole BCG, cell wall contituents of BCG, BCG-derived lipoarabidomannans, and other BCG components that are associated with the induction of an immune response, and 4) inactivated BCG by heat and formalin.

Chakraborty is alleged by the Examiner to teach that DCs that produce IL-12 efficiently stimulate T cells, whereas T cells that produce IL-10 are inhibitory and that DCs that produce IL-12 up-regulate co-stimulatory molecules CD80 and CD86.

The Examiner alleges that it would have been *prima facie* obvious for one of ordinary skill in the art at the time the present invention was made to add to GM-CSF plus IL-4 maturing agent as taught by Triozzi *et al.* and Labeur *et al.* BCG and interferon γ as taught by Bosch *et al.* in the method taught by Triozzi *et al.* and Labeur *et al.* for maturing DCs *in vitro* for use in producing an anti-cancer response because 1) a combination of BCG and interferon γ selectively produces more maturing DCs that secrete IL-12 than those inhibiting DCs secreting IL-10, as taught by Bosch *et al.*, 2) DCs that secrete IL-12 efficiently stimulate T cells, whereas DCs that produce IL-10 are inhibitory, as taught by Chakraborty *et al.*, 3) the ability of DCs to promote antitumor immunity correlates with their high efficiency of stimulating resting T cells and high production of IL-12, as taught by Labeur *et al.*

Applicant strongly disagrees with the allegations and assertions of the Examiner, but to further expedite prosecution claim 1 upon which claims 6 through 9 ultimately depend has been amended to recite "[a] method for producing an anti-tumor immune response comprising administration to an individual with a cancerous tumor a cell population comprising partially matured dendritic cells that have been induced to partially mature *in vitro*, wherein the partially matured dendritic cells take up and process antigen *in vivo* and are enabled to induce an anti-tumor immune response subsequent to administration to the individual". Such amendment is believed to point out with greater particularity the method comprises administering an individual

partially mature dendritic cells that have been induced to partially mature *in vitro* and that contact with antigen occurs *in vivo* subsequent to administration.

Further, as above, Triozzi *et al.* does not disclose or suggest of method of administering partially mature DCs that have not been contacted with antigen *in vitro*. In addition, Labeur *et al.* also do not disclose or suggest such a method. Further, Bosch *et al.* and/or Chakraborty *et al.* do not disclose or suggest any element missing from the teachings of Labeur *et al.* to render obvious any of claims 1 and 6-9. Even if either Bosch *et al.* and/or Chakraborty *et al.* were to teach or suggest those elements alleged by the Examiner above, any combination of those references with Triozzi *et al.* and/or Labeur *et al.* either alone or in any combination would not result in the present invention. If the references were combined as suggested by the Examiner, at most, the skilled artisan might use a maturation agent suggested by Bosch *et al.* to mature DCs that had been exposed to antigen prior to administration to a subject. That is not the invention as recited in any of claims 6 through 9. The addition of Chakraborty *et al.* which is alleged by the Examiner to teach the secretion of IL-12 by certain dendritic cells provides nothing that would disclose or suggest the present invention. As such, neither Triozzi *et al.* and/or Labeur *et al.* when considered alone or in any combination with Bosch *et al.* and/or Chakraborty *et al.* do not disclose or suggest the invention as recited in claims 6 through 9.

Applicant respectfully requests the Examiner to reconsider and withdraw the rejection of claims 6 through 9 as being unpatentable over Triozzi *et al.* in view of Labeur *et al.* and further in view of Bosch *et al.* and/or Chakrabory *et al.* in light of the amendments and remarks above.

Claims 14 through 18 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Triozzi *et al.* (above) in view of Labeur *et al.*, *J. Immunol.* 162:168-175, 1999 for reasons above. Further, the Examiner has admitted that Triozzi *et al.* does not teach the administration of DCs directly into the tumor, to a tissue area surrounding the tumor, into a lymph node directly draining a tumor area, directly to a circulatory vessel duct that delivers

blood or lymph to the tumor or a tumor afflicted organ, or into the circulatory system such that the cells are delivered to the tumor or tumor afflicted organ. The Examiner asserts that Labeur *et al.* teach that subcutaneous injection is not the optimal cell delivery system for *in vitro* generated DCs, at least in mice, because DCs migrate very inefficiently into the regional lymph nodes after subcutaneous injection. As such, the Examiner has alleged that it would have been *prima facia* obvious for one of ordinary skill in the art at the time the invention was made to replace subcutaneous injection of the DCs taught by Labeur *et al.* with administration of the DCs taught by Triozzi *et al.* and Labeur *et al.* directly into the tumor, to a tissue area surrounding the tumor, into a lymph node directly draining a tumor area, directly to a circulatory vessel duct that delivers blood or lymph to the tumor or a tumor afflicted organ, or into the circulatory system such that the cells are delivered to the tumor or tumor afflicted organ to increase the number of available sites for DC injection from which DCs could be readily delivered to the tumor in view of the allegation that DCs migrate inefficiently into the regional lymph nodes after subcutaneous injection, as taught by Labeur *et al.*

Applicant disagrees with the allegations and assertions of the Examiner, but to further expedite prosecution as set forth above claim 1 upon which claims 13 through 18 depend has been amended to recite "[a] method for producing an anti-tumor immune response comprising administration to an individual with a cancerous tumor a cell population comprising partially matured dendritic cells that have been induced to partially mature *in vitro*, wherein the partially matured dendritic cells take up and process antigen *in vivo* and are enabled to induce an anti-tumor immune response subsequent to administration to the individual". Such amendment is believed to point out with greater particularity the method comprises administering an individual partially mature dendritic cells that have been induced to mature *in vitro* and that contact with antigen occurs *in vivo* subsequent to administration.

Further, as above, Triozzi *et al.* and/or Labeur *et al.* when considered alone or in any combination do not disclose or suggest a method wherein partially matured dendritic cells are administered by any method. Further, contrary to the Examiners allegations, it would not have been obvious to one of ordinary skill to choose direct administration of the presently

claimed partially matured DCs over subcutaneous injection. Accordingly, Applicant respectfully requests that the rejection under 35 U.S.C. § 103(a) of claims 14 through 18 as being unpatentable over Triozzi *et al.* in view of Labeur *et al.* be withdrawn.

Claims 19 and 20 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Triozzi *et al.* (above) in view of Labeur *et al., J. Immunol.* 162:168-175, 1999 (*supra*), and further in view of Nikitina *et al., Int. J. Cancer* 94:825-833, 2001. The teaching of Triozzi *et al.* and Labeur *et al.* have been discussed above. The Examiner has admitted that Triozzi *et al.* and Labeur *et al.* do not teach that DCs are administered as an adjuvant to radiation therapy, chemotherapy or combinations thereof. In addition, the Examiner has admitted that Triozzi *et al.* and Labeur *et al.* do not teach that the allegedly partially matured dendritic cells are administered prior to, simultaneously with, or subsequent to radiation therapy, chemotherapy, or combinations thereof.

Nikitina *et al.* is alleged to teach that gamma irradiation induces the dramatic ability of DCs injected i.v. or s.c. to migrate and penetrate cancer tissue, and to take up apoptotic bodies, resulting in enhanced, potent antitumor responses. The Examiner has alleged that it would have been *prima facie* obvious for one of ordinary skill in the art at the time the invention was made to combine DC administration taught by Triozzi *et al.* and Labeur *et al.* with radiation therapy, because gamma irradiation induces the dramatic ability of DCs to migrate and penetrate cancer tissue, and to take up apoptotic bodies resulting in enhanced potent antitumor responses.

Applicant disagrees with the allegations and assertions of the Examiner. As set forth above, Triozzi *et al.* and/or Labeur *et al.* when considered either alone or in any combination do not teach the methods or compositions of the present claims. In particular, Triozzi *et al.* does not teach the administration of partially matured DCs, but only teaches the administration of immature DCs that lose their ability to induce an immune response when administered. In addition, the DCs taught in Labeur *et al.* are exposed to antigen *in vitro* prior to administration to an individual and are not the same as the DCs used in the presently claimed methods. Thus, Applicant submits that Triozzi *et al.* and/or Labeur *et al.* even if combined with

Nikitina *et al.* fail to teach or suggest each and every element of claims 19 and 20. Although Applicant does not believe that either Triozzi *et al.* and/or Labeur *et al.* teach the method or compositions set forth in the prior pending claims, but to further expedite prosecution, claim 1 upon which claims 19 and 20 depend has been amended to recite "[a] method for producing an anti-tumor immune response comprising administration to an individual with a cancerous tumor a cell population comprising partially matured dendritic cells that have been induced to partially mature *in vitro*, wherein the partially matured dendritic cells take up and process antigen *in vivo* and are enabled to induce an anti-tumor immune response subsequent to administration to the individual". Such amendment is believed to point out with greater particularity the claimed method comprises administering to an individual partially mature dendritic cells that have been induced to mature *in vitro* and that contact with antigen occurs *in vivo* subsequent to administration. Triozzi *et al.* and/or Labeur *et al.* when considered either alone or in any combination as set forth in greater detail above do not disclose or suggest such a method. Accordingly, Applicant respectfully requests that the rejection under 35 U.S.C. § 103(a) of claims 19 and 20 as being unpatentable over Triozzi *et al.* in view of Labeur *et al.* in further view of Nikitina *et al.* be withdrawn.

Claims 21 through 23, 25 and 27 through 32 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Triozzi *et al.*, (above) in view of Labeur *et al.* (above) and Sukhatme *et al.* (US 6,797,488). The Examiner has summarized the rejected claims as reciting the claimed compositions for administering: 1) *in vivo* directly into tumors, 2) into a tumor bed subsequent to surgical removal or resection of the tumor, 3) to a tissue area surrounding a tumor, 4) into a lymph node directly draining a tumor area, 4) into a circulatory vessel duct that delivers blood or lymph to the tumor, tumor bed, or a tumor afflicted organ, or 5) into the circulatory system such that the cells are delivered to the tumor, tumor bed, or tumor afflicted organ. The Examiner has viewed these limitations as a recitation of intended use and has therefore not given the limitations patentable weight when comparing the claims with the prior art. As such, the Examiner has alleged that claims 21-23, 25, and 27-32 read on the ingredient per se, which is a composition comprising dendritic cells partially matured *in vitro*.

As above, the teaching of Triozzi *et al.* and Labeur *et al.* are discussed. Further, the Examiner has alleged that Triozzi *et al.* teach that DCs generated *in vitro* by GM-CSF and IL-4 express the co-stimulatory molecules CD80 and CD86, and a low number of CD83. It is further alleged by the Examiner that Triozzi *et al.* teach that the amount of CDs generated is from 8.0×10^7 to 18×10^7. As such, the Examiner has concluded that the amount of DCs taught by Triozzi *et al.* is within the range of the claimed amount of DCs recited in claim 23. Further, the Examiner has admitted that Triozzi *et al.* and Labeur *et al.* do not teach DCs in a pharmaceutically acceptable carrier. Sukhatme *et al.* is cited by the Examiner as allegedly teaching an anti-angiogenic protein, fusion protein thereof and a composition thereof which comprises the protein combined with a pharmaceutically acceptable carrier. The Examiner alleges that although Triozzi *et al.* do not explicitly teach that the generated DCs are partially mature, the presently claimed DCs appear to be the same as those described in the prior art.

The Examiner has concluded from the above that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to combine the DCs taught by Triozzi *et al.* and Labeur *et al.* with a pharmaceutically acceptable carrier, as taught by Sakhatme *et al.*, for their storage.

Applicant must disagree with the rejection of the Examiner. The dendritic cells of the present invention are not the same as those of the cited prior art. In particular, the dendritic cells of Triozzi *et al.* are immature dendritic cells and are not partially matured dendritic cells as set forth in claims 21-23, 25 and 27-32. As set forth in the specification at page 9, line 28 through page 10, line 7 and page 11, lines 5 through 30, immature dendritic cells and partially mature dendritic cells differ in a number of ways including the levels of expression of a number of cell surface antigens, CD14, CD11c, CD80 and CD86, and in the phosphorylation level of a number of intracellular proteins including for example, jak2. Applicant respectfully directs the Examiner to additional differences in the cell surface phenotype and the levels of IL-10 and/or IL-12 produce by monocytic dendritic cell precursors cultured in the present of GM-CSF and IL-4 and those cultured in the presence of GM-CSF, IL-4 and a dendritic cell maturation agent. Immature dendritic cells induced to mature by the addition of, in this example, IFNγ and SAC

are clearly different in the amounts of IL-10 and/or IL-12 produced and in cell surface phenotype. As such, it is clear that the "partially mature" dendritic cells, immature dendritic cells contacted with a dendritic cell maturation agent, as recited in the present claims do not have the same properties as the dendritic cells of either Labeur *et al.* or Triozzi *et al.* Applicant also again respectfully directs the Examiner to page 2652, right column, lines 2 through 11 of Triozzi *et al.* where the authors conclude that the immature dendritic cells administered *in vivo* lost the co-stimulatory molecule B7-2 (CD86A) and showed a decrease in the intensity of CD11c suggesting the possibility that immunostimulatory activity typical of dendritic cells was down regulated. Applicant discloses in the specification as filed that the "partially matured" dendritic cells, as claimed, down regulate cytokine receptors on the surface as compared with "immature" dendritic cells making them less sensitive or responsive to any immunosuppressive effects of cytokines in the intratumoral space. Immature dendritic cells as defined in the specification include monocytic dendritic cells cultured in the presence of GM-CSF and IL-4. As such, the "partially matured" dendritic cells of claims 21 through 23 and 27 through 32 are not the same as those taught by Triozzi *et al.* Sukhatme *et al.* is cited by the Examiner as disclosing a pharmaceutical carrier. As Triozzi *et al.* and/or Labeur *et al.* do not teach the "partially mature" dendritic cells of the present invention or methods for their administration, the addition of the teachings of Sukhatme *et al.* does not disclose or suggest the present invention.

Claim 26 stands rejected under 35 U.S.C. § 103(a) as being unpatentable over Triozzi *et al.*, (above) in view of Labeur *et al.*, (above) and Murphy *et al.* (US 5,788,963, *supra*). The teachings of Triozzi *et al.* and Labeur *et al.* as alleged by the Examiner are set forth above. Further, the Examiner admits that neither Triozzi *et al.* or Labeur *et al.* teach that the generated DCs have been isolated from healthy individuals HLA matched to the individual to be treated. As such, the Examiner has cited Murphy *et al.* as allegedly teaching that DCs can be obtained from prostate cancer patients to be treated or from healthy individuals HLA-matched in terms of HLA antigens, because patients previously treated by radiation or chemotherapy often are not able to provide sufficient or efficient DCs. Murphy *et al.* is also cited by the Examiner as allegedly teaching that $CD8^+$ T cells, after interaction with antigen presenting cells, which

express MHC class I or class II molecules associated with the antigen, area sensitized and capable of killing any cells that express the specific antigen associated with matching MHC class I molecules.

Based on the above alleged disclosures the Examiner has opined that it would have been obvious to replace DCs obtained from the individual to be treated, taught by Triozzi *et al.* and Labeur *et al.* with DCs that have been isolated from a healthy individual HLA-matched to the individual to be treated, as taught by Murphy *et al.*, to increase the number of available DCs, for example, in situations where the patient to be treated cannot provide sufficient DCs, as taught by Murphy *et al.* Further, the Examiner has alleged that an HLA –matched DC would be necessary because antigen presentation of DCs in restricted to the complementing HLA molecule, also in view of the teachings of Murphy *et al.*

Applicant must again disagree with the Examiner. As above, the cited primary references Triozzi *et al.* as demonstrated in Labeur *et al.* fail to teach or suggest administration of partially mature DCs. The partially mature DCs as set forth in the present specification and claims have been induced *in vitro* to begin maturation, but have not progressed sufficiently through the maturation process to have substantially lost the ability to take up and process antigen. This is contrary to the teachings of Triozzi *et al.* where immature dendritic cells are administered to a patient, or Labeur *et al.* where DCs contacted with antigen and that have been induced to full maturation as indicated by their substantial loss of the ability to uptake antigen *in vitro* are administered to patients. As such, Triozzi *et al.* and/or Labeur *et al.* when either considered alone or in combination teach neither the administration of partially mature DCs or compositions that comprise partially mature DCs combined with a pharmaceutically carrier, much less the administration of partially mature DCs or compositions comprising partially mature DCs that have been HLA-matched to a patient to be treated as taught by Murphy. Therefore, any combination of Triozzi *et al.*, and/or Labeur *et al.* with Murphy *et al.*, do not teach or suggest each and every element of dependent claim 26.

In view of the above remarks Applicant respectfully requests the Examiner to reconsider and withdraw the rejection of claim 26 under 35 U.S.C. § 103(a) as being unpatentable over Triozzi *et al.*, (above) in view of Labeur *et al.*, (above) and Murphy *et al.* (US 5,788,963, *supra*).

Applicant notes that the Examiner has indicated that no claim are allowed. It appears that there is no rejection that pertains to claim 24 under either 35 U.S.C. §§ 102 or 103. As such it would appear that claim 24 should be considered allowable.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested. If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 206-467-9600.

Respectfully submitted,

Dated: 11 August 2009

By: Brian W. Poor

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Efficient Presentation of Soluble Antigen by Cultured Human Dendritic Cells Is Maintained by Granulocyte/Macrophage Colony-stimulating Factor Plus Interleukin 4 and Downregulated by Tumor Necrosis Factor α

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Summary

Using granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin 4 we have established dendritic cell (DC) lines from blood mononuclear cells that maintain the antigen capturing and processing capacity characteristic of immature dendritic cells in vivo. These cells have typical dendritic morphology, express high levels of major histocompatibility complex (MHC) class I and class II molecules, CD1, FcγRII, CD40, B7, CD44, and ICAM-1, and lack CD14. Cultured DCs are highly stimulatory in mixed leukocyte reaction (MLR) and are also capable of triggering cord blood naive T cells. Most strikingly, these DCs are as efficient as antigen-specific B cells in presenting tetanus toxoid (TT) to specific T cell clones. Their efficiency of antigen presentation can be further enhanced by specific antibodies via FcR-mediated antigen uptake. Incubation of these cultured DCs with tumor necrosis factor α (TNF-α) or soluble CD40 ligand (CD40L) for 24 h results in an increased surface expression of MHC class I and class II molecules, B7, and ICAM-1 and in the appearance of the CD44 exon 9 splice variant (CD44-v9); by contrast, FcγRII is markedly and sometimes completely downregulated. The functional consequences of the short contact with TNF-α are an increased T cell stimulatory capacity in MLR, but a 10-fold decrease in presentation of soluble TT and a 100-fold decrease in presentation of TT-immunoglobulin G complexes.

Dendritic cells (DCs)[1] play a critical role in antigen presentation in vivo (1). They exist in two stages of maturation. As immature cells, DCs are scattered throughout the body in nonlymphoid organs, where they appear to exert a sentinel function. They pick up and process antigen and subsequently move to the T-dependent areas of secondary lymphoid organs. During this process of maturation, they lose antigen-capturing capacity and become mature immunostimulatory DCs that trigger naive T cells recirculating through these areas (2, 3).

A similar maturation process occurs spontaneously when Langerhans cells (LCs, which represent immature DCs in skin) are cultured in vitro (4, 5). Under these conditions LCs rapidly lose the capacity to pick up and process soluble antigen, but acquire high T cell costimulatory capacity. Thus, both in vivo and in vitro studies suggest that antigen capture/processing and immunostimulation are the property of DCs at different stages of maturation.

The mechanism of antigen uptake determines the efficiency of presentation of soluble antigens on class II molecules (6). The pinocytic activity of DCs has been reported to be at least as high as that of other APCs (7). In addition, splenic DCs and LCs express FcγRII, which is lost when these cells mature in vitro (4, 8). Although DCs are able to present soluble antigen (5, 9–11), their efficiency has not been compared to that of other APCs. A general consensus has emerged that DCs may actually be rather inefficient in presenting soluble antigens and it has been argued that, for this reason, efficient presentation in vivo may be an exclusive property of antigen-specific B cells (12).

A common progenitor for granulocytes, macrophages, and DCs has been identified in mouse bone marrow (13) as a MHC class II negative cell that can develop into the three different myeloid pathways under the aegis of GM-CSF. Proliferating precursors of DCs that can be expanded into DC lines in vitro with GM-CSF are present both in mouse bone marrow (14) and peripheral blood (15). To date, human DC lines have

[1] *Abbreviations used in this paper:* CD40L, CD40 ligand; DCs, dendritic cells; Ii, invariant chain; LCs, Langerhans cells; TT, tetanus toxoid.

been generated only from $CD34^+$ precursors isolated from cord blood (16) or bone marrow (17) using a combination of GM-CSF and TNF-α.

There are several reasons for wishing to establish in vitro cultures of immature DCs. First, to exploit their antigen-presenting capacity; second to compare them with other APCs for this function; third, to identify the signals that modulate antigen capturing and presenting function. Here we describe a method to culture DCs from human peripheral blood, such that the phenotypic and functional characteristics of immature DCs are retained. These cells are indeed as efficient as antigen-specific B cells and can use FcγRII to further increase uptake of antigen in antigen–antibody complexes. Maturation of these cells can be induced by TNF-α, resulting in upregulation of their capacity to stimulate naive allogeneic T cells and downregulation of their capacity to present soluble antigen.

Materials and Methods

Media and Reagents. The medium used throughout was RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 50 μg/ml kanamycin, 5×10^{-5} M 2-ME (Gibco Laboratories, Grand Island, NY) and 10% FCS (Hyclone Laboratories, Inc., Logan, UT). Tetanus toxoid (TT) was purchased from Connaught Laboratories, Ltd. (Willowdale, Ontario, Canada). Human anti-TT antibodies (all IgG) were purified by chromatography on protein A–Sepharose from concentrated culture supernatants of EBV-B cell clones (18). Human recombinant IL-2, IL-4, and GM-CSF were produced in our laboratory by PCR cloning and expression in the myeloma expression system described by Baumeister et al. (19). The concentration of IL-4 and GM-CSF were determined using commercial ELISA assays. Purified human recombinant GM-CSF and TNF-α were a generous gift of Dr. Manfred Brockhaus (Hoffmann-La Roche, Basel, Switzerland). A soluble chimeric fusion protein between the mouse CD8 α-chain and the human CD40 ligand (CD40L) was a generous gift of Dr. Peter Lane (Basel Institute for Immunology) (20).

Culture of DCs from Peripheral Blood. PBMC were isolated on lymphoprep cushions (LSM; Organon Teknika Corp., Rockville, MD) resuspended in RPMI-10% FCS, and allowed to adhere to 6-well plates (Costar Corp., Cambridge, MA). After 2 h at 37°C the nonadherent cells were removed and the adherent cells were detached by incubation with Mg^{2+} and Ca^{2+} free PBS containing 0.5 mM EDTA at 37°C as described (21). In some experiments, PBMCs were separated on multistep Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and the light density fraction from the 42.5–50% interface was recovered and depleted of $CD19^+$ B and $CD2^+$ T lymphocytes using magnetic beads (Dynal, Oslo, Norway). The adherent or light density fractions were cultured at 3×10^5/ml in RPMI–10% FCS supplemented with 50 ng/ml GM-CSF and 1,000 U/ml IL-4.

FACS® Analysis. Cell staining was performed using mouse monoclonal antibodies followed by FITC- or PE-conjugated affinity purified, isotype-specific goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, AL). The following mAbs were used: L243 (IgG2a, anti-DR), W6/32 (IgG2a, anti-MHC class I); 32.2 (IgG1, anti-FcγRI), AT10 (IgG1, anti-FcγRII), OKT3 (IgG2a, anti-CD3) (all from the American Type Culture Collection, Rockville, Maryland); SPVL3 (IgG2a, anti-DQ, provided by Dr. H. Spits, DNAX, Palo Alto, CA); B7.21 (IgG1, anti-DP, provided by Dr. J. Trowsdale, ICRF, London, UK); BU45 [IgG1, anti-invariant chain (Ii); provided by Dr. N. Koch, Institute of Immunology and Genetics, DKFZ, Heidelberg, Germany]; NA1/34 (IgG2a, anti-CD1a), WM25 (IgG1, anti-CD1b), 10C3 (IgG1, anti-CD1c) (all provided by Dr. F. Calabi, Hammersmith Hospital, London, UK); B7.24 (IgG2a, anti-B7; provided by Dr. Mark Deboer, Immunogenetics, Ghent, Belgium); RR1/1 (IgG1, anti-ICAM-1), TS1/22 (IgG, anti-LFA1), TS2/9 (IgG1, anti-LFA3) (all provided by Dr. T. Springer, Harvard Medical School, Boston, MA); Leu 11a (IgG1, anti-CD16), Leu M3 (IgG2b, anti-CD14), Leu M5 (IgG2b, anti-CD11c), Leu M9 (IgG1, anti-CD33), Leu 11c (IgG1, anti-FcγRIII) (Becton Dickinson & Co., Mountain View, CA); 25.34 (IgG, anti-CD44), 11.24 (IgG1, anti CD44-v9) (all provided by Dr. C. Mackay, Basel Institute for Immunology); G28.5 (IgG1, anti-CD40, provided by Dr. A. E. Clark, University of Washington, Seattle, WA); HD37 (IgG1, anti-CD19; Boehringer Mannheim, Mannheim, Germany). The samples were analyzed on a FACScan® (Becton Dickinson) using propidium iodide to exclude dead cells.

MLR. 1.5×10^5 responding cells either from allogeneic adult PBMCs or cord blood (allogeneic MLR) or autologous PBMCs (autologous MLR) were cultured in 96 flat-bottom microplates (Costar Corp.) with different numbers of irradiated (3,000 rad from a ^{137}Cs source) stimulator cells (DC or PBMC). Thymidine incorporation was measured on day 5 by a 16-h pulse with [^{3}H]thymidine (1 μCi/well, sp act, 5 Ci/mMol; Amersham Life Science, Buckingham, UK).

Antigen Presentation Assays. TT-specific EBV-B cell clones and TT-specific T cell clones were isolated and maintained as previously described (18). T cell clone AS11.15 recognizes a TT determinant corresponding to residues 947–967 in association with DP4. To measure the efficiency of presentation of a soluble antigen, 2×10^4 TT-specific T cells were cultured with 5×10^3 cultured DCs (3,000 rad), 2×10^4 irradiated autologous EBV-B cells (6,000 rad) or 10^5 autologous PBMCs (3,000 rad) in the presence of different concentrations of TT in 200 μl RPMI–10% FCS in flat-bottom microplates. The cultures were set up in the presence or absence of a fixed concentration of a mixture of six different anti-TT IgG antibodies (0.5 μg/ml each). [^{3}H]Thymidine incorporation was measured after 48 h. TT and anti-TT antibodies were allowed to react for 1 h before the addition of APCs and T cells. In some experiments, total PBMCs (1.5×10^5) or polyclonal short-term TT-specific T cell lines (2×10^4) were used as a source of TT-specific cells. [^{3}H]Thymidine incorporation was measured on days 5 and 2, respectively.

Results

Culture Conditions for the Generation of DCs with Antigen-presenting Capacity. Adherent cells or the light density Percoll fraction from PBMCs were depleted of T and B cells and cultured in RPMI–FCS supplemented with various combinations of GM-CSF, IL-4, and TNF-α. The cell yields, surface phenotype and functional properties of cells grown with different cytokine combinations are shown in Table 1. It is evident that a combination of GM-CSF and IL-4 provided the best conditions for the generation of cells with the characteristic phenotype and functional properties of DCs (high expression of CD1, class II and B7, and high stimulatory capacity in allogeneic and autologous MLR). Furthermore cells from GM-CSF + IL-4–dependent cultures were the most efficient at presenting soluble antigen TT to specific T cell

clones. Cells grown with a combination of GM-CSF and TNF-α (16) were inferior to those obtained with GM-CSF + IL-4, especially for presentation of soluble antigen. We therefore used DCs from GM-CSF + IL-4–dependent culture in subsequent experiments.

In a typical experiment, after ~7 d of culture with GM-CSF + IL-4, 50–80% of the cells appear as loosely adherent clumps or isolated floating cells with the typical dendritic morphology (see an example in Fig. 1) and motility, as assessed by time lapse videorecording (data not shown). Analysis of surface markers (Table 1 and Fig. 2) showed that the large cells were homogeneous and expressed high levels of MHC class I and class II molecules, CD1a, CD1b and CD1c, FcγRII, ICAM-1, CD11b, CD11c, CD40, B7, and CD33. CD14 was either low or negative in different preparations. Furthermore, DCs were positive for Ii, LFA-1, LFA-3, and CD44 and negative for FcγRI and FcγRIII (see also Table 2). Anti-CD3 and anti-CD19 antibodies were always used as control and found negative. Cell growth rapidly slowed down after the first 3–4 wk, but viable cells could be maintained in culture with occasional feeding for up to 3 mo.

Stimulatory Capacity of DCs. Cultured DCs were compared with PBMCs for their capacity to stimulate alloreactive T cells. Different numbers of DCs or PBMCs from the same donor were cultured with a fixed number of allogeneic T cells. Fig. 3 *a* shows that as few as 50 DCs could trigger a substantial response; on a per cell basis, DCs were 300-fold more effective than PBMCs in stimulating adult T cells. It is interesting to note that only DCs but not PBMCs could trigger cord blood T cells (Fig. 3 *b*). The observation that cord blood T cells, which are entirely naive, could be stimulated only by DCs underlines the specialized role of DCs for T cell priming.

Efficient Presentation of Soluble Antigen and Antigen–Antibody Complexes by Immature DCs. To evaluate the capacity of DCs to present a soluble antigen, we compared DCs, PBMCs, and antigen-specific B cells for their capacity to present TT to a TT-specific T cell clone. To evaluate the possible effect of FcγR in enhancing capture of antigen–antibody complexes, the cultures were set up in the presence or absence of a fixed concentration of anti-TT IgG antibodies.

As evident from Fig. 4, the efficiency of presentation, as measured from the TT concentration necessary to give 50% of maximum response, varies with the type of APC. DCs (Fig. 4 *a*) were the most effective APCs, since they could present TT at a concentration of 10^{-10} M, while PBMCs (Fig. 4 *b*) and nonspecific B cells (Fig. 4 *c*) required antigen concentrations higher than 10^{-8} M. Furthermore, in the presence of anti-TT antibodies, the efficiency of DCs increased at least 100-fold and a significant proliferative response was

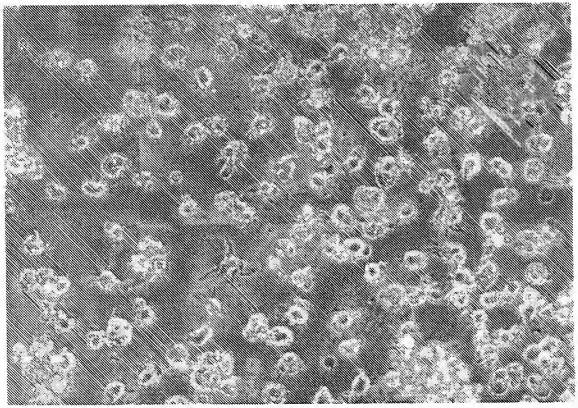


Figure 1. Typical appearance of DC cultures on day 15.

Table 1. *Cell Yield, Surface Phenotype, Stimulatory, and Antigen-presenting Capacity of Light Density Mononuclear Cells Cultured with Various Cytokine Combinations*

	Alone	GM-CSF	IL-4	TNF-α	GM-CSF TNF-α	GM-CSF IL-4
Cell yield (%)*						
Day 4	23	68	23	43	57	84
Day 8	6	114	25	46	74	99
Day 20	2	152	14	64	90	125
Surface markers						
CD1a	–	–	–	–	±	+
CD1b	–	±	–	–	±	+
CD1c	–	–	–	–	±	+
DR	++	++	++	++	++	++
DQ	–	–	+	–	+	+
Class I	++	++	++	++	++	++
FcγRII	++	+	++	++	+	+
B7	–	±	–	+	+	+
CD40	–	++	+	+	++	++
CD11c	+	++	++	+	++	++
ICAM-1	+	++	+	++	++	++
CD14	+	±	±	+	–	–
Stimulation and antigen presentation‡						
Allogeneic MLR‖						
Maximum response	nd	32 ± 2§	40.9 ± 2.7	33.4 ± 1.7	53.1 ± 3	68.1 ± 2.2
No. cells for 50% response		6,000	4,000	12,000	3,000	600
Autologous MLR‖						
Maximum response	nd	<1	<1	<1	<1	10.1 ± 2
TT presentation¶						
Maximum response	nd	89.4 ± 4.5	nd	nd	8.2 ± 2.1	134.6 ± 4
ng/ml TT 50% response		100			>10³	10

* Cell yield at days 4, 8, and 20 expressed as percent of input cells.
‡ The various cell populations were tested on day 8 for their capacity to stimulate in allogeneic and autologous MLR. The maximum response and the number of cells required for stimulating 50% of the maximum response are shown.
§ 5 × 10³ cells from the various cell populations were tested on day 10 for their capacity to present different concentrations of TT to a TT-specific T cell clone. The maximum response and the concentration of TT required for stimulating 50% of the maximum response are shown.
‖ cpm × 10^{-3} ± SD.

obtained at TT concentrations of 10^{-14} M. When compared with PBMCs, DCs were at least 100-fold more efficient, both in the absence and in the presence of anti-TT antibody (Fig. 4 *b*). Finally, DCs were as efficient as some antigen-specific B cells (Fig. 4 *c*) and, in the presence of soluble antibodies, appeared to be the most efficient APC for soluble antigens.

We also compared DCs and PBMCs for their capacity to present TT to autologous peripheral blood T cells and to polyclonal short-term TT-specific lines. As evident from Fig. 5, *a* and *b*, DCs were again more powerful than PBMCs, both in terms of maximum response and amount of TT required. The shape of the dose-response curve may be due to the presence of T cells with different sensitivities to antigen and thus may reflect the ability of DCs to stimulate a higher number of specific T cells.

Modulation of Surface Phenotype and Antigen-presenting Function by CD40L and TNF-α. The above results indicated that DCs obtained from GM-CSF + IL-4 cultures shared many prop-

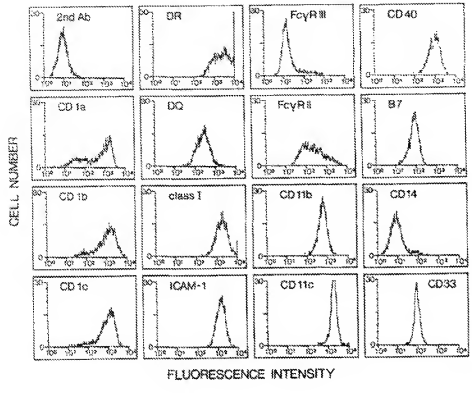
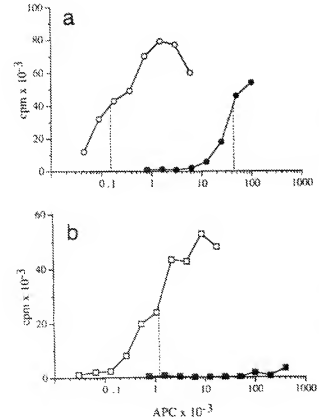


Figure 2. Surface phenotype of DCs at day 8 of culture. The histograms show fluorescence values on gated large cells. The amplification was set at a very low value (×400) to allow quantitative measurement of the extremely bright cells. The first panel shows the control staining with isotype-matched irrelevant antibody.



erties with immature DCs such as LCs, at least with respect to surface phenotype and presentation of soluble antigen. We therefore asked whether these cells might be induced to mature in culture and whether this maturation would affect antigen-presenting capacity.

We tested the effect of TNF-α and CD40L, which represent two effector molecules of natural and acquired immunity. As shown in Table 2, DCs underwent a rapid change in surface phenotype upon incubation with TNF-α or CD40L. By 24 h, surface MHC class I and class II molecules increased two- –threefold, while Ii expression was reduced by approximately half. ICAM-1 expression also increased, a fact that was presumably responsible for the spontaneous cell aggregation observed. B7 and CD40 were also upregulated, whereas FcγR.II was rapidly and sometimes completely downregulated. Interestingly, treatment with TNF-α increased CD44 expression and induced the appearance of a new splice variant carrying exon 9 (22).

Some of these changes resemble those occurring in vivo when LCs move from skin to lymph nodes (4). We therefore asked what consequence these changes might have on the capacity of DCs to stimulate T cells and to present soluble

Figure 3. Cultured DCs are highly stimulatory in MLR and are the only cells capable of triggering cord blood T cells. Adult peripheral blood (●) or cord blood mononuclear (▮) cells were cultured with different numbers of allogeneic PBMCs (●, ▮) or DCs (○, □) from the same donor. The proliferative response was measured on day 5.

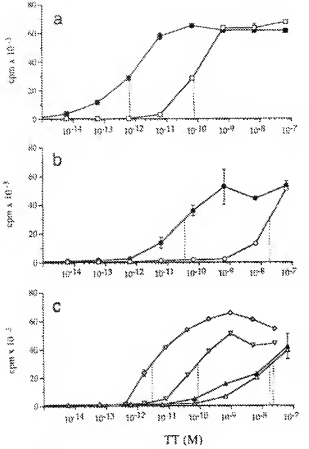


Figure 4. Cultured DCs are the most efficient APCs for presentation of soluble antigen as such or complexed with IgG antibodies. The three panels represent the proliferative response of the same T cell clone to different concentrations of TT in the presence or absence of a fixed concentration of anti-TT IgG antibodies and different irradiated APCs. (*a*): 5×10^3 DCs in the absence (□) or presence (■) of anti-TT antibody. (*b*): 10^5 PBMCs in the absence (○) or presence (●) of anti-TT antibody. (*c*): 2×10^4 EBV-B cells from two TT-specific clones (◇, ▽) in the absence of anti-TT antibody or a nonspecific polyclonal line in the absence (△) or presence (▲) of anti-TT antibody. Proliferative response was measured on day 2.

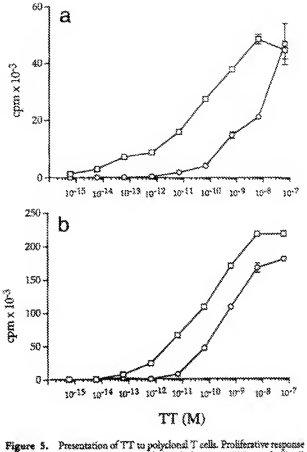


Figure 5. Presentation of TT to polyclonal T cells. Proliferative response of peripheral blood T cells (*a*) or polyclonal short-term TT-specific T cell lines (*b*) to different concentrations of TT presented by autologous mononuclear cells (○) or autologous DCs (□). Proliferative response was measured on days 5 and d 2, respectively. The backgrounds of the response without TT (autologous MLR) were subtracted.

antigen. Pretreatment with TNF-α or CD40L increased two- -threefold the stimulatory capacity in MLR (data not shown), a result that is consistent with the expression of higher levels of MHC products, adhesion, and costimulatory molecules. Remarkably, the same DCs showed a reduced capacity to present TT after treatment with TNF-α (Fig. 6 *a*), being about 10-fold less efficient. Moreover, the enhanced presentation of immune complexes was completely abrogated, concomitant with a downregulation of FcγRII (Fig. 6 *b*).

TNF-α did not have cytotoxic effect on GM-CSF + IL-4–dependent DCs and actually, in some cases, slightly enhanced cell viability. However, the downregulation of FcγRII and the decrease in antigen-presenting capacity were irreversible, since they were still present even several days after TNF-α had been removed (data not shown).

Discussion

The availability of immature DCs is instrumental for studying the mechanisms of antigen capture and processing by these cells, as well as to identify signals that modulate this function. In this study we have shown that it is possible to grow in vitro human cell lines with many of the characteristics of immature DCs. The two most striking findings are the highly efficient presentation of soluble antigen by these cell lines, and their rapid response to TNF-α leading to up-regulation of adhesion and costimulatory molecules and down-regulation of antigen-capturing and -processing capacity.

Our DC lines differ from those described by Caux et al. (16) in two important aspects: the use of adult PBMCs (23) rather than cord blood precursors and the use of IL-4 rather than TNF-α. Indeed adult adherent cells grown with GM-CSF + TNF-α have lower stimulatory capacity and are unable to present soluble antigen (Table 1), a fact that can be explained by the capacity of TNF-α to regulate antigen-presenting function (Fig. 6). Our DC lines were generated from adult peripheral blood and require IL-4 in addition to

Table 2. *Phenotypic Changes of DCs Cultured for 24 h with TNF-α or CD40L*

	GM-CSF + IL-4	+ TNF-α*	+ CD40L‡
Second Ab	28§	33	30
CD1a	369	492	428
CD1b	200	423	270
CD1c	295	454	377
DR	1,555	2,938	2,117
DQ	549	1,416	1,210
DP	129	281	187
Ii	245	185	126
MHC class I	991	2,044	1,796
FcγRII	1,684	737	862
B7	84	163	127
CD40	673	1,612	742
CD11c	534	414	481
ICAM-1	228	620	457
LFA-1	550	448	517
LFA-3	219	290	252
CD44	845	1,235	1,274
CD44-v9	62	147	98
CD14	75	40	40

* DCs were incubated for 24 h with 10 ng/ml TNF-α.
‡ DCs were incubated for 24 h with CD40L-mouse CD8 at a saturating concentration as determined by staining with anti-mouse CD8.
§ Mean fluorescence intensity. Comparable results were obtained in four separate experiments.

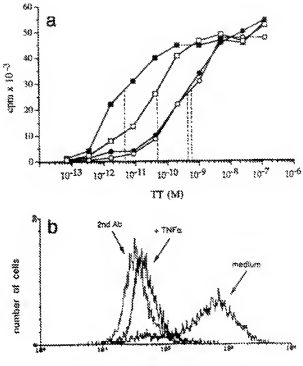


Figure 6. Treatment of cultured DCs with TNF-α downregulates FcγRII and decreases antigen-capturing and -presenting capacity. (*a*) Proliferative response of a TT-specific T cell clone to different concentrations of TT in the presence of 5×10^4 irradiated DCs that have been pretreated with TNF-α (○, ●) or left untreated (□, ■). The culture were set up in the absence (○, □) or in the presence (●, ■) of anti-TT IgG antibodies as in Fig. 4. (*b*) surface expression of FcγRII on DCs that had been pretreated overnight with TNF-α or left in growth medium.

GM-CSF to maintain the immature, antigen presentation competent state. These conditions have been previously shown to increase CD1 expression on adherent cells (21, 24). It is not clear what role IL-4 may play, but it is interesting to speculate it may antagonize the effect of TNF-α and other maturation-inducing signals. Whether IL-4 or other cytokines may play a physiological role in maintaining the immature DC pool in vivo is a matter of speculation.

The identification of GM-CSF/IL-4–expanded cells as DCs was based on three well-established and accepted criteria (25, 26): first, their typical morphology and motility; second, their surface phenotype, with high expression of CD1, MHC class I and class II, Ii, FcγRII, B7, CD40, ICAM-1, LFA-3, and CD11c; and third, their high stimulatory capacity for naive T cells (27–29). In this regard, it is worth noting that only DCs but not PBMCs could activate cord blood T cells. This finding reinforces the notion that DCs are the only cells capable of triggering naive T cells and is an apparent contrast with a report that adult $CD45RA^+$ naive T cells can be stimulated by allogeneic PBMCs (30). It is possible, however, that either cord blood cells have higher requirements for costimulation than adult T cells, or that the alloreactive response by adult $CD45RA^+$ T cells may involve memory cells that have reverted to the $CD45RA^-$ phenotype, but retain the capacity to respond to nonprofessional or semiprofessional APCs (31, 32).

Presentation of soluble antigen by DCs has been reported to be rather inefficient in the sense that relatively high concentrations of antigen were required (10^{-6} and 10^{-7} M), comparable to those required by other nonantigen-specific APCs (4, 9–11). In contrast, we found that DCs cultured with GM-CSF + IL-4 can present TT at concentrations of 10^{-10} M and are therefore 100–300-fold more efficient than nonspecific B cells or PBMCs. These DCs are actually comparable to antigen-specific B cells, which can use membrane Ig for antigen capture. Furthermore, in the presence of immune complexes, DCs become even more efficient than antigen-specific B cells, being able to present TT at the extraordinary low concentration of 10^{-12} M. This is the most efficient presentation of soluble antigen reported to date.

This highly efficient presentation of soluble antigen depends on the preservation of the immature phenotype, since

it is lost when DCs are induced to mature by TNF-α. It is thus possible that the antigen-presenting capacity of DCs has been previously underestimated because the immature $FcγR^+$ cells may have been lost or induced to mature during the isolation procedure.

Several mechanisms may contribute to the efficient antigen presentation of DCs: first, their capacity for clustering T cells in an antigen-independent fashion (33); second, the expression of high levels of MHC molecules, allowing presentation of more T cell determinants; third, the high expression of adhesion and costimulatory molecules and the low surface charge (34, 35), which may lower the number of determinants required for T cell activation (36, 37); and fourth, the high level of fluid-phase pinocytosis (7) and the expression of functional FcγR (11, 38).

A striking finding is the response of DCs to TNF-α and CD40L. Within 24 h, surface expression of MHC class II, ICAM-1, LFA-3, CD40, and B7 increases two- to threefold, whereas expression of Ii and FcγRII decreases. The functional consequences are an increased T cell stimulatory capacity in MLR, but a 10-fold decrease in presentation of soluble TT and a 100-fold decrease in presentation of TT-IgG complexes. The effect on class II molecules and Ii is of particular interest. Preliminary experiments indicate that the increase in surface class II expression is not accompanied by an increase in class II biosynthesis, suggesting an effect of TNF-α at the post-translational level. Indeed, staining for intracellular class II molecules revealed that TNF-α induces a rapid disappearance of class II containing structures that are prominent in immature DCs and a redistribution of class II molecules towards the cell periphery (Sallusto, F., unpublished results). Thus, there are differences in the mechanism that lead to down-regulation of antigen presenting capacity in cultured DCs and in fresh LCs. Whereas in both cases FcγRII is down-regulated, a downregulation of class II synthesis is observed only in LCs (39, 40). Further work is required to identify the level of this regulation and the signals involved (41, 42).

It is interesting to discuss our results in the context of the well known maturation pathway of LCs. It has been shown that LCs form a reservoir of immature DCs that, upon antigenic stimulation, resume their migratory behavior and move to the draining lymph nodes, where they arrive as mature DCs (1, 43). A similar maturation process is known to occur spontaneously when these cells are cultured in vitro (4). On the basis of our results it is interesting to hypothesize that TNF-α may play a physiological role in vivo in the induction of migration and maturation (44, 45). Local production of TNF-α at sites of encounter with "infectious" antigen (46) may induce maturation of DCs and their migration from tissues into secondary lymphoid organs. It is interesting to note that TNF-α induces the appearance of a CD44 isoform carrying the v9 exon, which may be involved in controlling migratory behavior (47). The role of CD40L may be limited to a later stage, when DC localize in T-dependent areas of lymph nodes.

Whereas local production of TNF-α may play a physiological role in regulating antigen presentation by DCs, high systemic levels of TNF-α, such as in malignancies or chronic inflammatory diseases, may be detrimental (48). Too much TNF-α may cause generalized immunosuppression by inducing all DCs to mature and lose the capacity to present new incoming antigens.

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